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Restriction and Enhancement of Human Immunodeficiency Virus Type 1 Replication by Modulation of Intracellular Deoxynucleoside Triphosphate Pools

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Human immunodeficiency virus type 1 (HIV-1) replication is shown to be sensitive to the intracellular concentration of deoxynucleoside triphosphate substrates. Addition of thymidine to established cell lines resulted in a dramatic reduction of virus production. The effect could be substantially alleviated by addition of deoxycytidine, which, alone, enhanced viral titers by a factor of 2 to 3. Hydroxyurea treatment abolished HIV-1 replication in peripheral blood mononuclear cells and could be reversed by deoxyadenosine. These data show that HIV-1 replication occurs under suboptimal DNA precursor conditions.

Retroviral reverse transcription takes place within a porous nucleoprotein structure made up of viral *gag* proteins in the cytoplasm (4). This structure allows the diffusion of essential cofactors such as deoxynucleoside triphosphates (dNTPs) and magnesium cations necessary for replication. In mammalian cells, dNTP concentrations are highly asymmetric, fluctuate during the cell cycle, and can vary greatly between cell types (6, 8, 24, 27, 28). For lymphoid cell lines, the intracellular concentrations are usually in the order dGTP ≤ dCTP ≤ dATP ≤ TTP while the dCTP/TTP ratio can vary by a factor of 2 to 10 (8, 10).

Deoxyribonucleotides are derived from scavenging of deoxynucleosides from without, and reduction of ribonucleoside diphosphates from within, the cell. Ribonucleotide reductase is the key enzyme involved in the balanced regulation of dNTP pools. It catalyzes the reduction of ribonucleoside diphosphates (CDP, ADP, GDP, and UDP) to the corresponding deoxyribonucleoside diphosphates and is allosterically regulated by TTP, dATP, and dGTP (31). Addition of deoxyribonucleosides to the culture medium may alter intracellular dNTP pools. Thus, addition of thymidine (dThd) results in a 5- to 10-fold reduction of dCTP and a corresponding increase in TTP levels (10). Accordingly, the asymmetry of dNTP pools, or even the relative dearth of dCTP alone, might influence the kinetics of viral replication. It has been shown that addition of hydroxyurea (HU), a potent inhibitor of ribonucleotide reductase, can reduce vaccinia virus replication in a dose-dependent manner by several orders of magnitude. This effect could be reversed by addition of deoxyadenosine (dAdo) and correlated with dNTP pool changes (33). Furthermore, dThd addition restricted Rous sarcoma virus replication in cultured cells although the basis for this effect was unclear (35).

Our interest in the regulation of intracellular dNTP pools stems from the fact that highly biased dCTP/TTP ratios may lead to increased mutation frequency in mammalian cells, as has been shown for the adenosine phosphoribosyltransferase locus (25, 30). In the case of viruses, this may result in more defective progeny as well as altering overall viral replication kinetics. It has been suggested that G→A hypermutation of the human immunodeficiency virus (HIV) genome is associated with dNTP pool imbalances (38, 39). Here it is shown that dNTP concentrations can influence HIV type 1 (HIV-1) production.

Manipulation of intracellular dNTP pools. Before evaluating the effect of dNTP pool changes on HIV replication, the sensitivity of the established CD4-positive T-cell lines to exogenous nucleosides was established to confirm that the cell lines used behaved in a way anticipated from known metabolic regulatory pathways. Table 1 shows the effect of dThd, deoxycytidine (dCyd), and dThd plus dCyd on the intracellular dNTP pools for the three cell lines used for subsequent HIV infections. The data show that the dNTP pools in these cell lines behave in the manner expected. (i) The dNTP pools were highly asymmetric with dCTP or dGTP being the lowest. The dCTP/TTP ratios were of the order of 1:2 to 1:6. (ii) Addition of dThd decreased dCTP and increased TTP. An eightfold decrease in the dCTP/TTP ratio was achieved by the addition of 50 μM dThd which had little effect on the NTP pools (see Table 2) and could be abrogated by the addition of dCyd.

Restriction of HIV-1 replication by dThd and reversion by dCyd. The sensitivity of HIV replication towards dThd was investigated with the established cell lines H9 and U937. Cells were treated with increasing dThd concentrations prior to infection with virus derived from the infectious clone of HIV-1 LAI (29). In order to decrease drug toxicity, cells were washed 5 h postinfection and the culture was continued in drug-free medium. Subsequent virus production was assessed by reverse transcriptase (RTase) activity in the culture supernatants. While the addition of 10 μM dThd decreased HIV production only in H9 cells, 50 μM practically abrogated replication in both H9 (Fig. 1A) and U937 (Fig. 1B) cell lines.

As shown in Table 1, the intracellular dCTP depletion following dThd addition could be reversed by the addition of dCyd. Addition of 5 μM dCyd with 100 μM tetrahydrouridine

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TABLE 1. Nucleoside treatment affects intracellular dNTP pools[a]

Cell line	Treatment	dNTP level (nmol/10^9 cells)			
		dCTP	TTP	dATP	dGTP
U937		8	50	28	20
U937	10 μM dThd	6	56	21	20
U937	50 μM dThd	2	123	11	23
H9		23	58	33	34
H9	10 μM dThd	18	140	44	43
H9	50 μM dThd	17	300	79	113
H9	10 μM dThd + 5 μM dCyd	49	184	31	78
H9	50 μM dThd + 5 μM dCyd	68	176	58	80
H9	1 μM dCyd	40	60	41	36
H9	2 μM dCyd	40	86	33	43
H9	5 μM dCyd	39	44	47	32
H9	10 μM dCyd	49	70	34	35
H9	20 μM dCyd	56	57	41	34
CEM		9	35	14	7
CEM	10 μM dThd	8	44	13	10
CEM	50 μM dThd	5	119	12	29
CEM/Myc		5	12	7	3
CEM/Myc	10 μM dThd	4	12	10	3
CEM/Myc	50 μM dThd	3	10	9	3

[a] dNTP pool levels were determined by high-pressure liquid chromatography after overnight treatment with the nucleosides as previously described (12). Values shown are those of a typical experiment with less than 20% variation. CEM/Myc cells were CEM cells which were identified as mycoplasma contaminated during a routine screening (7). To inhibit dCyd deamination when treating cells with exogenous dCyd, 100 μM of the cytidine deaminase inhibitor, THU, was always used in addition to dCyd.

(THU, a dCyd deaminase inhibitor used to prevent degradation of dCyd) to either H9 or U937 infected cells was able to substantially reverse the inhibitory effect of dThd (Fig. 1C and D).

Enhancement of HIV-1 replication by dCyd. Interestingly, the presence of dCyd on its own seemed to enhance replication of HIV. Figures 2A and B show that HIV production was influenced by dCyd with maxima at 5 and 2 μM dCyd for U937 and H9 cells, respectively. Both the reversion of dThd-mediated HIV inhibition and the enhanced virus production in the presence of dCyd correlated with a dNTP pool change towards balancing the different levels. To investigate this phenomenon further, 5 μM dCyd plus 100 μM THU were maintained throughout the culture, as opposed to an initial 5-h incubation. One-half of the culture medium was changed three times a week and replaced with fresh medium and dCyd plus THU. This resulted in an increase of HIV production (Fig. 2C). As judged by RTase activity, HIV productivity was enhanced by a factor of 3. However, addition of dCyd might be affecting HIV replication simply by changing cellular growth properties. With the methyl-tetrazolium dye reduction assay (MTT) (26), no change in cell growth or survival was noted between dCyd-plus-THU-treated and untreated H9/LAI cultures, supporting a direct relationship between an augmented intracellular dCTP and HIV replication.

dThd does not down-regulate CD4 or affect proviral transcription. While it would appear that dThd was influencing HIV replication via modulation of the intracellular de-

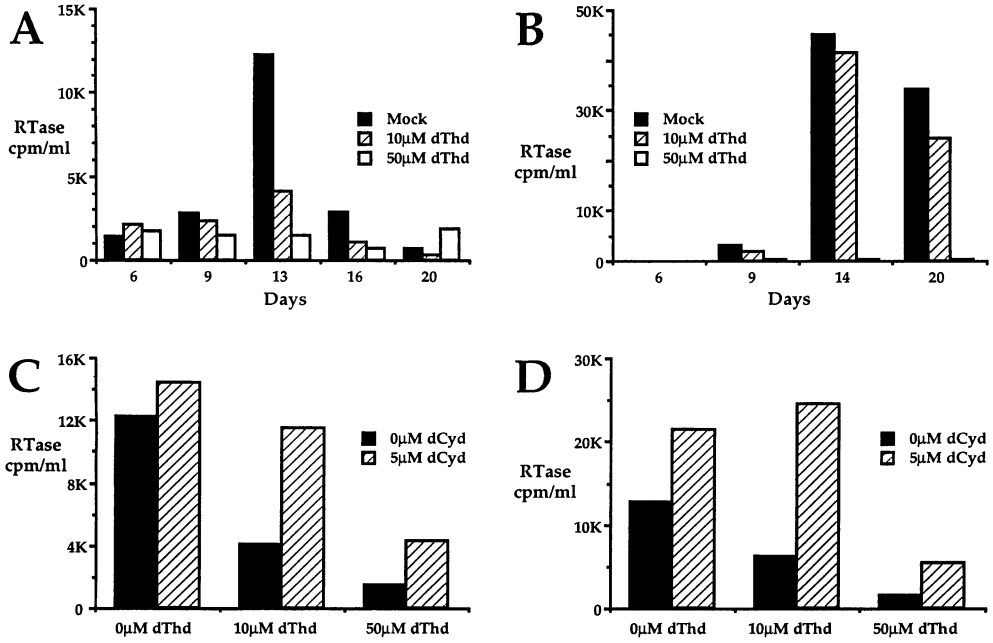

FIG. 1. Inhibition and reversion of HIV-1 replication in cell lines by pyrimidine nucleosides. The restriction of HIV-1 replication by dThd on the H9 (A) and U937 (B) cell lines, as well as reversion of the effect by a combination of dThd and dCyd (plus 100 μM THU) in H9 (C) and U937 (D) cells, is shown. Supernatants of SW480 cells transfected by the calcium phosphate method with the infectious clone pLAI (formerly called pBRU-2 [29, 40]) were used as a source of HIV-1. Cultures were infected with 1×10^4 to 2×10^4 cpm of RTase activity per 10^6 cells. RTase activities of pelleted culture supernatants were determined as a function of time (A and B) or 13 days postinfection (C and D). Cells were treated with dThd (Sigma) alone or in combination with dCyd (Sigma) overnight prior to infection. The infection was then carried out at 37°C with drugs still present. After 2 h of incubation, the remaining virus was washed away and the cells were incubated for another 5 to 7 h with drugs before a change to normal media. In all experiments involving dCyd, 100 μM THU, a dCyd deaminase inhibitor (Calbiochem), was used to prevent degradation of dCyd. The human lymphocyte and monocyte cell lines CEM, H9, ACH-2, U937-2, U1, and MT-2 were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. The human CD4-negative adenocarcinoma cell line SW480 was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. All cell lines were periodically screened for mycoplasmas with Hoechst 33258 dye (7).

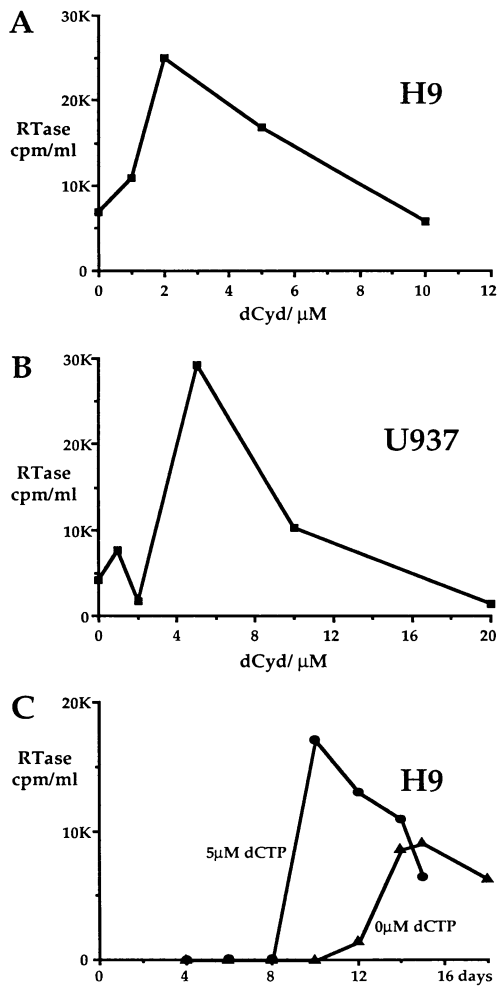

FIG. 2. (A and B) Prior dCyd treatment of H9 (A) and U937 (B) cells enhances HIV-1 replication. RTase activities of pelleted culture supernatants at day 14 postinfection were determined as a function of dCyd concentration. dCyd was present overnight before, and 5 h after, infection. (C) Continual dCyd treatment of H9 cells enhanced HIV-1 replication. One-half of the culture medium was changed three times a week and replaced with fresh medium and dCyd plus 100 μM THU.

oxynucleotide pools, it might be possible that the drug is inhibitory to HIV at other stages, notably entry or transcription. To exclude a simple down-regulation of cell surface CD4 receptor, H9, CEM, and U937 cells were treated with 10 to 100 μM dThd for 3 to 12 h; treatment was followed by fluorescence-activated cell sorting. No decrease in CD4 receptor density was noted for any of the cell lines (data not shown).

A possible effect of dThd on proviral transcription was investigated by activating the latently infected cell lines, ACH-2 and U1, by phorbol myristate acetate in the presence of dThd. HIV-1 was recovered and titered for syncytia on MT-2 cells. No change in virus titer was observed (data not shown), demonstrating that proviral transcription by RNA polymerase II was not affected. The above controls are consistent with the idea that dThd modulated HIV-1 replication by influencing dNTP, and most profoundly dCTP, pools (Table 1).

HU, but not dThd, restricts HIV-1 replication on PBMC. Having shown a dThd-dependent restriction of HIV-1 replication in T-cell lines, the drug was used to little avail on HIV-1-infected peripheral blood mononuclear cells (PBMC). Addition of up to 10 mM dThd resulted in only partial restriction of HIV-1 replication (<30%, data not shown). This is to be contrasted with the 50 μM necessary to totally inhibit HIV-1 replication on T-cell lines (Fig. 1A and B) and might reflect a heightened catabolism of dThd in activated primary T lymphoblasts. In order to show that dNTP pools could restrict HIV-1 replication in PBMC, another drug, HU, was used. HU interacts directly with ribonucleotide reductase, probably acting as a scavenger for the free radical intermediates formed during ribonucleoside diphosphate reduction (31). Treatment results in >90% reduction in dATP pools, 40 to 80% reduction in dCTP and dGTP pools, and even an increase in TTP levels (9, 33).

HIV-1 infection was carried out under such conditions that HU was present mainly during proviral synthesis. HIV-1 was adsorbed to PBMC at 4°C for 30 min and then shifted to 37°C, leading to virus-cell fusion within minutes (11). Drugs were added for the following 5 h, during which reverse transcription should be taking place. Under these conditions, 3 mM HU showed almost complete virus inhibition (Fig. 3A). Addition of dAdo, known to reverse dNTP pool changes mediated by HU, resulted in near-normal levels of HIV-1 production (Fig. 3B). HU-induced cytotoxicity of phytohemagglutinin-stimulated PBMC has been observed ex vivo when millimolar concentrations were used (36). During the 5-h treatment postinfection, HU cytotoxicity was presumably minimal as evidenced by the complete reversal of restricted HIV-1 replication by dAdo.

Mycoplasmas reduce intracellular dNTP pools and HIV-1 replication. During routine screening of CEM cultures, one was found positive for mycoplasma contamination. As mycoplasmas secrete a pyrimidine-specific nucleosidase (32), the possibility of restricted HIV replication on mycoplasma-contaminated cell lines via dNTP precursor depletion was investigated. Tables 1 and 2 show that mycoplasma contamination resulted in a two- to threefold reduction of intracellular dNTP and NTP pools, while the addition of exogenous dThd had no effect whatsoever on the intracellular dCTP or TTP concentrations. Equal numbers of CEM cells with and without mycoplasma contamination, yet derived from the same cell clone, were infected with an equal volume of virus from the same stock. HIV-1 replication was restricted 100-fold (Fig. 4). Inhibition of the RTase assay (21, 22) can be ruled out as pelleted virus was used. Not surprisingly, given the results in Tables 1 and 2, addition of dThd had no effect on the culture. These data suggest that HIV replication was restricted by the mycoplasmas competing for, or degrading, nucleoside substrates in the culture medium.

It is perhaps not surprising that the kinetics of HIV-1 replication was sensitive to dNTP pools, given the data for vaccinia virus (33). That inhibition of HIV-1 replication by either dThd or HU was reversible by compounds known to restore the dNTP pool perturbations, notably dCyd and dAdo, respectively, is probably the best evidence that the inhibitory effect of the two compounds occurs via modulation of dNTP pools. Certainly in the case of dThd, no effect could be found at the level of cell surface CD4 density or proviral transcription by RNA polymerase II. We have sought in vain a quantitative effect of dThd on proviral synthesis. This was due to the contamination of the viral stock (derived from transfection of SW480 cells by plasmid DNA) by residual plasmid DNA or newly formed proviral DNA from a few dying cells. Despite filtration and DNase treatment of the virus stock, there was always a residual PCR signal in heat-inactivated virus sample used as a control. However, in long-term cultures treated with

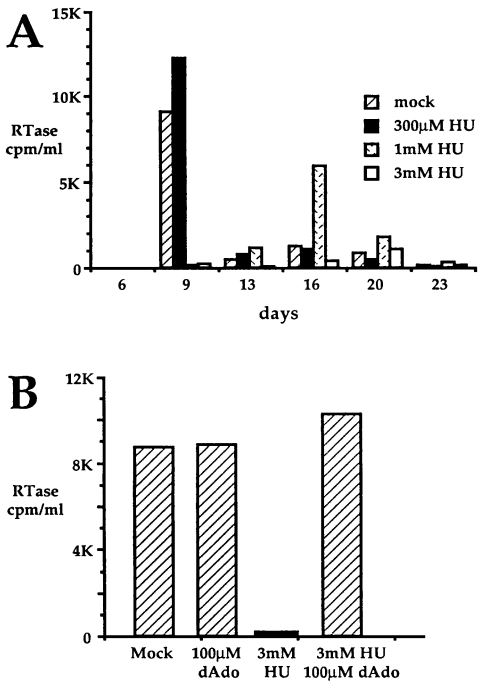

FIG. 3. Inhibition and reversion of HIV-1 replication in PBMC by HU and dAdo. The effect of HU on HIV-1 replication in PBMC (A) and that of the combination of HU and dAdo (B) are shown. RTase activities of pelleted culture supernatants are given as a function of time (A) or peak RTase activity (B) postinfection. PBMC from healthy seronegative blood donors were separated on a Ficoll-Hypaque (Pharmacia) density gradient and depleted of monocytes by adherence in the culture flasks. The nonadherent cells were harvested, and the PBMC thus obtained were stimulated with 2.5 μg of phytohemagglutinin (Pharmacia) per ml supplemented with 5 U of recombinant interleukin 2 (Amersham) per ml for 2 to 5 days prior to infection or cocultivation. The PBMC lymphoblasts were treated with HU and dAdo such that the drugs were present mainly during the reverse transcription step immediately after virus entry. This was necessary because a simple procedure to control for potential effects on polymerase II-mediated transcription was lacking for primary cells. Precooled PBMC and virus were mixed and kept on ice for 30 min. After temperature shift to 37°C and incubation for a further 15 min, free virus was washed away. Cells were then cultured in medium containing the different drugs for 5 h. The medium was changed, and the culture was continued under normal conditions.

dCyd, there must be a net increase in the kinetics of proviral synthesis. This is suggested by the detection of virus earlier, as well as by higher titers in the culture supernatant (Fig. 2C). The converse situation, i.e., reduced proviral synthesis upon depletion of dNTP pools, seems tenable in the light of this and other data (Fig. 1, 3, and 4 and Table 1). Perhaps the use of dCyd and THU to augment HIV-1 yield may be of some practical use, for example, in the preparation of Western blot (immunoblot) strips or in making high-titer virus stocks.

Intracellular dNTP pools are significantly reduced in resting, as opposed to phytohemagglutinin-stimulated, PBMC, with typical values being on the order of dCTP ~ 0.3 pmol, TTP ~ 0.2 pmol, dATP ~ 2.4 pmol, and dGTP ~ 0.4 pmol per 10^6 cells (8). By contrast, the corresponding values for lymphoblasts are 3- to 90-fold greater, i.e., dCTP ~ 3 pmol, TTP ~ 18 pmol, dATP ~ 6 pmol, and dGTP ~ 2 pmol per 10^6 cells (34, 37). Furthermore, two- to fourfold differences in dNTP levels have been noted, depending on the cell cycle (6, 8, 24, 27, 28).

TABLE 2. Nucleoside treatment affects intracellular NTP pools[a]

Cell line	Treatment	NTP level (nmol/10^9 cells)			
		CTP	UTP	ATP	GTP
CEM		490	880	7,330	440
CEM	10 μM dThd	680	1,126	6,486	470
CEM	50 μM dThd	493	833	5,700	400
CEM/Myc		200	240	3,890	190
CEM/Myc	10 μM dThd	233	293	3,806	226
CEM/Myc	50 μM dThd	133	160	2,800	147

[a] NTP pool levels were determined by high-pressure liquid chromatography after overnight treatment with the nucleosides as previously described (12). Values shown are those of a typical experiment with less than 20% variation. CEM/Myc cells were CEM cells which were identified as mycoplasma contaminated during a routine screening (7). To inhibit dCyd deamination when treating cells with exogenous dCyd, 100 μM of the cytidine deaminase inhibitor, THU, was always used in addition to dCyd.

Given that relatively small changes in dCTP can reduce HIV-1 replication (Fig. 1; Table 1), it is probable that dNTP concentrations would severely restrict, if not preclude, HIV-1 replication in resting T lymphocytes. Indeed, the unusual, partial RNA-DNA structures seen upon infection of resting human T cells probably represent incomplete reverse transcripts restricted by the dearth of dNTPs (44, 45). Virus could be recovered from cultures harboring these incomplete structures if they are stimulated by a mitogen within 14 days postinfection. Upon activation, dNTP pools would increase, resulting in completion of the provirus, and hence productive infection.

Given the substantial body of data showing that modulation

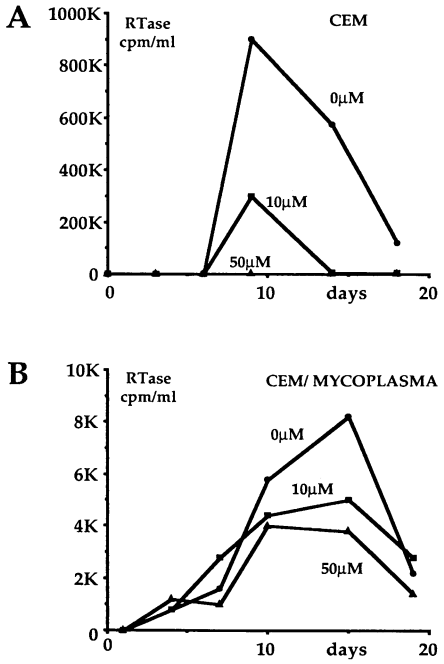

FIG. 4. Restriction of HIV-1 replication, and abrogation of dThd modulation, by mycoplasma infection. Addition of 10 or 50 μM dThd restricted or abolished HIV-1 replication (A), whereas in the presence of mycoplasma-infected CEM cells (B), the differences between dThd-treated and untreated cultures are not significant. Virus was derived from pLAI. In this experiment, air-dried filters were counted with scintillation fluid, which explains the higher RTase values compared with those in the other figures.

of dNTP pools and their relative ratios has profound effects on replication fidelity, an increase in the formation of defective progeny following dThd and HU treatment cannot be excluded (1, 5, 13, 14, 16–20, 23, 25, 41–43). The fact that dThd treatment could not be completely reversed by dCyd suggested, as mentioned above, that addition of dThd resulted in complex phenomena. However, the data in Fig. 3 are more straightforward, with complete reversion of 3 mM HU-induced inhibition by addition of 100 μM dAdo. In this experiment, cells were treated for the first 5 h postinfection and then washed to remove HU. This treatment not only reduced HIV-1 replication but increasingly displaced the peak from day 9 (0 and 300 μM HU) to day 16 (1 mM HU) to day 20 (3 mM HU) (Fig. 3A). Were there simple restriction of HIV-1 replication, proviral synthesis would be expected to be rapidly completed once the drug was removed, as treated cells recover from the effects of both dThd and HU within 0.5 to 1 h (10). Thus, dNTP misincorporation, resulting in defective proviral formation, might explain the shift in peak height from day 9 to day 20.

Retroviruses are uniquely sensitive to the mutagenic effects of dNTP precursor imbalances. The intracellular concentrations of NTPs are high and, by virtue of their involvement in metabolism and signal transduction, effectively buffer RNA viruses from the effects of NTP imbalances. DNA viruses, on the other hand, are sensitive to the fluctuations of dNTP pools. However, replication within the nucleus or cytoplasm is subject to proofreading by the 3′ exonuclease activity of the viral DNA polymerase and cellular or viral proofreading enzymes. In contrast, reverse transcriptase has no 3′ exonuclease activity and only plus-strand DNA synthesis is subject to proofreading by cellular enzymes after proviral translocation to the nucleus. The manipulation of replication fidelity has been suggested as a novel means to handle RNA viruses. Indeed, the remarkable G→A hypermutation associated with retroviruses (38) might reflect transient dCTP depletion (39). In view of this and the above data, the prospect of using dThd or HU as an anti-HIV drug may be considered.

The half-life of millimolar concentrations of dThd in peripheral blood was on the order of 50 to 100 min, essentially because of efficient renal clearance (3, 12, 46), and while of some use in cancer therapy, dThd was considered too impractical because of the large quantities and prolonged course of treatment necessary to kill malignant cells. HU has been used to treat chronic myelogenous leukemia with some success (2, 15). Concentrations in plasma of 1 to 3 mM could be achieved by oral administration; however, the half-life of HU in plasma was variable and on the order of 2 to 4 h (2, 15). Side effects included myelosuppression, particularly granulocytopenia, beyond millimolar levels in plasma (36). The problem with these cytostatic molecules, as opposed to HIV-specific drugs for therapeutic purposes, will be the specific targeting of the infected cell. By interfering with lymphocyte proliferation, a prerequisite for HIV replication, such drugs might restrict immune responses to the very pathogens to which the lymphocytes had responded.

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